

Claims

1. A process for the mutagenesis of a double-stranded polynucleotide sequence (master sequence) of n base-pairs having a (+)-strand and a complementary (-)-strand comprising the steps
 - (i) creation of a collection of single-stranded fragments of the (+)-strand of the master sequence wherein all members of the collection have the same 5'-terminus and have a deletion in the 3'-terminus such that the collection represents (+)-strands with a length of n-1, n-2, n-3, nucleotides;
 - (ii) introduction of at least one universal or degenerate nucleotide at the 3'-terminus of the (+) strand produced in step (i);
 - (iii) elongation of the (+)-strand produced in step (ii) to the full length of the master sequence using the (-)-strand or fragments thereof as a template strand for the elongation;
 - (iv) synthesis of a (-)-strand by using the (+)-strand produced in step (iii) as a template strand thereby effecting mutations in the (-)-strand at the positions of the previous universal or degenerate nucleotides compared to the master sequence.
2. A process according to claim 1, wherein the collection of single-stranded fragments in step (i) is created by incorporating nucleotide analogs and subsequent cleavage in alkaline or acidic solution.
3. A process according to claim 2, wherein the nucleotide analog is an alpha-phosphothioate nucleotide and oxidative cleavage is achieved by iodine at the phosphothioate bonds.
4. A process according to claim 1, wherein step (ii) comprises the elongation of the collection of single stranded fragments produced in step (i) with universal base or degenerate base by enzymatic or chemical methods.
5. A process according to claim 4, wherein terminal deoxynucleotidyl transferase or DNA polymerases or DNA/RNA ligases are used for elongation.
6. A process according to claim 1, wherein deoxyinosine, 3-nitropyrrole, 5-nitroindole or a nucleotide analog with promiscuous base pairing property is used as a universal nucleotide in step (ii).
7. A process according to claim 1, wherein N⁶-methoxy-2,6-diaminopurine (K), N⁶-methoxy-aminopurine (Z), hydroxylaminopurine (HAP), 2'-deoxyribonucleoside

triphosphate (dyTP), 6H,8H-3,4-dihydropyrimidol [4,5-c][1,2] oxazin-7-one (P), N⁴-aminocytidine, N⁴-hydroxy-2'-deoxycytidine, N⁴-methoxy-2'-deoxycytidine, 8-oxodeoxy-guanosine triphosphate (8-oxo-G) or a nucleotide analog with promiscuous base pairing property is used as degenerate nucleotide in step (ii).

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8. A process according to claim 1, wherein an oligonucleotide of the general formula

$$p(U)_a(N)_b^*(S)_c[TERM]$$

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with

p = 5'-phosphate or hydroxy-group or any chemical group capable of forming diester bonds

U = universal or degenerate bases

a = arbitrary integral number from 0 to 10000

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N = mixture of four bases (A/T/G/C (standard nucleotides))

b = arbitrary integral number from 0 to 100

* = cleavable group such as phosphothioate bonds in phosphothioate nucleotides

S = standard nucleotide or nucleotide analog

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c = arbitrary integral number from 0 to 100

[TERM] = a dye terminator or any group preventing elongation of the oligonucleotide, with the proviso that a+b>0,

is used in step (ii) to introduce universal or degenerate bases to the collection of single-stranded fragments created in step (i).

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9. A process according to claim 8, wherein the oligonucleotide is designed in a way that

(a) stop codons and/or

(b) amino acids which disrupt secondary structures,

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being avoided in the collection of the mutagenized polynucleotide sequences.

10. A process according to claim 8, wherein the oligonucleotide is designed in a way that

(a) transition mutations or

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(b) transversion mutations,

being effected in the collection of the mutagenized polynucleotide sequences.

11. A process according to claim 8, wherein the single-stranded fragment created in step (i) which is not ligated with the oligonucleotide is removed using exonuclease.

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12. A process according to claim 1, wherein the elongation in step (iii) is effected by a PCR reaction.
13. A process according to claim 1, wherein step (iii) comprises the synthesis of a (-) 5 single stranded plasmid polynucleotide sequence from a double-stranded plasmid harboring the master sequence using a primer which anneals downstream of the (+)-strand of the master sequence, and annealing of this (-)-ss- plasmid polynucleotide sequence with the (+)-strand produced in step (ii), and elongation of the (+)-strand.
- 10 14. A process according to claim 1, wherein step (iii) comprises the synthesis of a (-) -single-stranded plasmid harboring the master sequence using a primer which anneals downstream of the (+)-strand of the master sequence in the presence of uracil and standard nucleotides and after the elongation of the (+)-strand produced in step (ii), the uracil carrying (-)-single-stranded plasmid is digested with uracil glycosylase.
- 15 15. A process according to claim 1, wherein a PCR amplification is used after step 20 (iii) in order to synthesize a (-)-strand complementary to the (+)-strand produced in step (iii), thereby effecting a double-stranded master sequence carrying mutations.